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The finding of homozygous deletions has been useful in localizing new tumor-suppressor genes and identifying the involvement of known suppressor genes. Four major approaches can identify homozygous deletions: a candidate gene approach where losses of a particular sequence are sought, a candidate positional search where a genome scanning strategy utilizes spaced markers, a candidate locus where markers of a known homozygous deletion of one tumor system are used to specify a region for investigation in a second tumor type, and an unbiased approach such as the representational difference analysis (RDA). Approaches utilizing RDA, candidate genes, and candidate loci in breast cancer are currently under investigation by our group. Using the candidate gene approach, a homozygous deletion of the DPC4 gene was identified within a breast cancer cell line. This suggested the involvement of this TGF-β-like pathway in breast cancer, and has stimulated the analysis of other candidate genes that may be potential members of this pathway. This approach, as well as the RDA and candidate locus methods, will contribute to our understanding of homozygous deletions and the suppressive pathways involved in this cancer.

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FOREWORD

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Scott Kern

PI - Signature Date

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Introduction

A rational approach to the diagnosis and treatment of breast cancer should be enabled through the understanding of precisely how the cancer cells differ from normal cells. The most definitive and most basic means to accomplish this lies in the definition of the genetic mutations which distinguish the DNA of cancer from that of normal cells. Currently, very few tumor-suppressor genes are known to be mutated during the growth of human cancers, and the role of most putative proto-oncogenes has remained unclear. The reasons are rather straightforward. The identification of tumor-suppressor genes has largely depended on the identification of deletions, but most losses and gains of chromosomal material in adult tumors involve very large chromosomal areas, limiting the ability to specify the target gene.

A new and powerful technique termed the Representational Difference Analysis (RDA) is especially geared toward identifying genetic deletions, amplifications, and rearrangements regardless of their size. For the first time, a procedure readily allows the definition of the smallest genetic lesions, those which should more rapidly aid the discovery of key genes. We have already shown the utility of this approach, defining a candidate subregion of BRCA2, 24-fold smaller than the initially reported region (1,2). For this IDEA grant, we proposed to explore the use of RDA in breast cancer to identify small homozygous deletions and rearranged DNA fragments.

Additional approaches are now available as well, and the cooperative use of these techniques promises to considerably accelerate the speed of new discovery. One approach is the use of the newly available closely-spaced markers throughout the genome to perform genome-scanning for homozygous deletions, which unlike RDA can be targeted to regions of special interest. Also available is the identification of new candidate genes and signaling pathways which provide markers to test specific hypotheses about potential new tumor-suppressor sites in cancer.

Body

Methods

Procedures of the representational difference analysis (RDA) were performed essentially as described by Wigler and colleagues, as modified by our laboratory (1, 3)(below). PCR assays to detect homozygous deletions were performed as described by us (1, 2, 4). An assay for the presence of the STS (sequence-tagged site) is used to test the original genomic DNAs for the absence of template in the neoplasm; a negative result in the tumor thus is considered to indicate a presumptive homozygous deletion. Confirmation of the homozygous deletion is done by testing of a second nonoverlapping STS.

Briefly, RDA is performed as follows. Amplicons are generated by restriction endonuclease digestion of genomic DNA, followed by the ligation of PCR anchors to each 5' end to serve as primer sites for "whole-genome PCR". When used to clone deleted fragments, the driver amplicon is generated from xenograft or cell line DNA, the tester amplicon from normal DNA. When used to clone sites of rearrangement, the driver amplicon is generated from normal DNA, and the tester amplicon from xenograft or cell line DNA. One or both amplicon(s) is size-fractionated on an agarose gel to isolate the smaller fragments, in order to ensure the reduction of genome complexity. The anchor primers of the tester amplicon are then removed and replaced with a second primer site, to allow the selective amplification of tester DNA in later steps. The amplicons are mixed together, with a vast excess of driver amplicon (500 ng vs. 40 μg) present as a saturated solution (5 μl total). The DNA mixture is melted by boiling and allowed to anneal. Unannealed (single-stranded) tester and driver DNA is degraded by mung bean nuclease. Homohybrids of tester DNA are selectively and exponentially amplified by PCR. This constitutes one round of RDA. Subsequent rounds of RDA start with

replacement of the anchor primers, and use increasingly smaller ratios of tester to driver amplicon in the hybridization, always with driver DNA in saturated solution. For amplicons generated with BamHI or BglII, two to three rounds are sufficient to produce distinct bands when the difference product of the round is evaluated on agarose gel electrophoresis.

Samples for RDA analysis included two breast cancers of patients of the Johns Hopkins Hospital, propagated as xenografts in nude mice and stored frozen. Normal tissues of each patient were snap frozen, and served as control normal tissue. Twenty-three cell lines available from the ATCC (American Type Culture Collection) were grown in tissue culture. DNA was extracted from xenografts, human tissues, and cell lines using standard techniques.

Results and Discussion

RDA was initiated on the two xenografts of human breast cancer. We initially had difficulties with the RDA of one of the samples, while the other appeared to give adequate amplicons. We determined that a technical barrier could be produced by the presence of single-stranded DNA within the starting material. DNA prepared from additional xenograft tissue of this case proved adequate for use, and contained minimal amounts of single-stranded DNA. Rounds of RDA were successfully performed, and characterizations of the RDA products are underway.

An opportunity presented itself to rapidly survey for additional homozygous deletions at a novel locus not known at the time of the initial grant application. We had identified a novel tumor-suppressor gene in pancreatic cancer, DPC4, which is at chromosome 18q21.1 (4). A major mechanism of its inactivation was by homozygous loss in tumors. A survey of the 23

ATCC cell lines for sequence changes and homozygous deletions of DPC4 was undertaken. The MDA-MB468 cell line was found to have a homozygous deletion of DPC4 (5). This suggested the involvement of the DPC4 pathway in breast cancer, presumed to involve a TGF-β-like signaling pathway involving cell surface receptors, SMAD proteins (such as DPC4, that mediate such signals in both invertebrates and vertebrates), and the activation of transcription of genes in the nucleus. With this lead, the status of a number of TGF-β superfamily receptors was targeted for homozygous deletion screening and sequence analysis. This survey of TGF-β superfamily pathway candidate members is underway.

Homozygous deletion loci, identified from studies of other tumor types but where the gene target had not yet been specified, were increasingly shown to be important in the study of breast cancer. The PTEN gene, for example, has its highest rate of homozygous deletions in gliomas, but homozygous deletions also occur in breast cancer. We have extended our studies of breast cancer to include high resolution genome scanning of candidate loci at such sites identified from other tumor systems.

Conclusions

Novel tumor-suppressor genes continue to be identified in breast cancer through the study of homozygous deletions. This approach, to identify homozygous deletions in breast cancer and to screen breast cancers for candidate sites of homozygous deletions, appears to be one of the more efficient means to discover novel and important regulatory systems that are impaired in human breast cancers.

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